Assignment of the ^{31}P and ^{1}H resonances in oligonucleotides by two-dimensional NMR spectroscopy

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The use of new ^{1}H-detected heteronuclear ^{1}H-^{31}P shift correlation experiments is demonstrated for oligonucleotides of 12 and 40 base pairs. The methods give unambiguous assignments of the ^{31}P resonances and also permit identification of the C4′ and C5′ sugar protons. Use of the new methods enables one to make sequence-specific resonance assignments without reference to a known or assumed conformation of the DNA fragment.

Oligonucleotide *^{31}P-NMR* *2D NMR* *Shift correlation* *Poly(dA-dT)*

1. INTRODUCTION

^{31}P NMR can provide important structural and dynamic information on nucleic acids [1]. A problem in using this information originates in the difficulty of resolving and assigning the individual resonances in the ^{31}P NMR spectra. So far, application of modern 2D NMR methods to solving this problem has been limited to the study of relatively small oligonucleotide sequences [2–4]. Here it is demonstrated that ^{1}H-detected correlation techniques can be used successfully to correlate ^{1}H and ^{31}P chemical shifts for oligonucleotides of up to 40 base pairs. ^{1}H-detected heteronuclear shift correlation techniques have recently been shown to provide a gain in sensitivity of one to two orders of magnitude when applied to the study of ^{13}C and ^{15}N [5,6]. For ^{31}P NMR, this gain in sensitivity is substantially lower because of the relatively high magnetogyric ratio of ^{31}P. Nevertheless, a gain in sensitivity by a factor of about three is easily obtainable over 2D experiments that employ direct ^{31}P detection.

2. METHODS AND RESULTS

Two slightly different schemes have been used in the present study and are sketched in fig.1. The scheme of fig.1a provides a 2D absorption mode spectrum and consequently offers the highest spectral resolution. The scheme of fig.1b utilizes the 'constant-time concept' [7,8], optimizing magnetization transfer from ^{31}P to ^{1}H and maximizing sensitivity of the method. First we will discuss the application of scheme 1a to the study of the dodecamer d(CATGGATm^{5}CCATG) duplex. Rather than transferring ^{1}H magnetization into ^{1}H-^{31}P multiple quantum coherence [2,3] we prefer to start with ^{31}P magnetization directly. In principle, one would expect a loss in sensitivity by a factor of γ_H/γ_P ($\approx$2.5) of scheme 1a vs the multiple quantum experiment. However, generating the

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Abbreviations: 2D, two-dimensional; TMP, trimethylphosphate; TSP, trimethylsilylpropionate; m^{5}C, 5-methylcytosine

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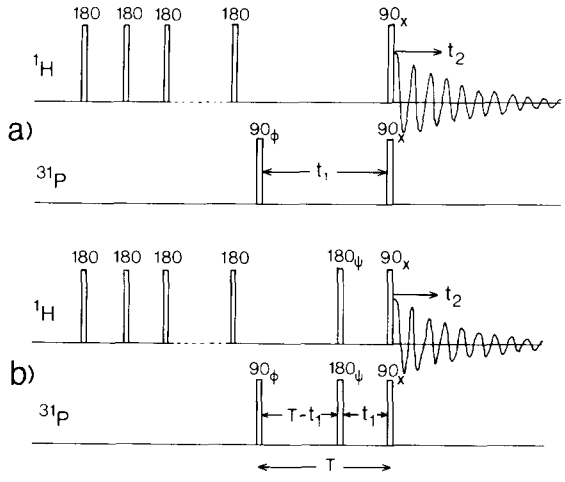

Fig.1. Pulse schemes for generating 2D ^{1}H-^{31}P correlation spectra. During the delay period between consecutive scans, both schemes use a series of 40 180° pulses, spaced by 50 ms, to presaturate ^{1}H resonances. In both schemes, the phase ϕ of the first ^{31}P pulse is cycled $x, y, -x, -y$ and the receiver is cycled $x, -x, x, -x$. In addition, the phase ψ of the 180° pulses in scheme b is incremented by 90° every 32 scans; furthermore, each time ψ is incremented, the phase of the receiver is inverted. Data in odd- and even-numbered scans are stored separately and processed in the standard manner to separate positive and negative modulation frequencies. Scheme a results in a 2D pure absorption spectrum whereas scheme b requires an absolute value mode calculation in the t_1 dimension. Dispersive data in the t_2 dimension are discarded after the first Fourier transformation and the final spectrum is absorptive in this dimension.

^{1}H-^{31}P multiple quantum coherence is not an effective process and in practice scheme 1a appears to be more efficient. The detected ^{1}H magnetization transferred from ^{31}P at $t_2 = 0$ is in antiphase with respect to ^{31}P but in phase with respect to all other protons. Absorption mode 2D spectra can therefore be recorded readily without the need for z filters or purge pulses [2]. Signals that do not originate from transfer from ^{31}P are suppressed by phase cycling and by presaturation of the ^{1}H signals using a series of 180° pulses in between experiments.

Fig.2 shows the 2D correlation spectrum of the dodecamer. In addition to the expected three-bond connectivities to H3′ and H5′, H5′′ also all of the four-bond H4′-C4′-C5′-O-P connectivities are observed. Since most of the C4′ protons are resolved and assignable by means of a 2D NOE experiment, sequential assignment information follows directly from this type of spectrum. We have found the presence of P-H4′ connectivity in all other oligonucleotides studied with this method so far. It may therefore be expected that this important connectivity is a general feature observed in this type of spectrum.

In scheme 1a, the amount of ^{31}P magnetization transferred to ^{1}H is a function of the length of the evolution period, t_1. The t_1 duration for which maximum transfer occurs depends on the sizes of the J_{HP} couplings and on the ^{31}P transverse relaxation time. In practice, a maximum is reached for a t_1 duration of about 25 ms. Scheme 1b exploits this fact by keeping the length of the evolution period fixed to a time, T (≈25 ms), and moving the pair of $^{1}H/^{31}P$ 180° pulses stepwise through this period. This makes it appear as if the evolution period is varied from $-T$ to $+T$, i.e. it gives an effective acquisition time of $2T$ in the t_1 dimension. Note that the detected ^{1}H signals are modulated as a function of t_1 only by the ^{31}P chemical shifts and not by scalar coupling, providing the highest possible resolution in the t_1 dimension. Unfortunately, the overall duration of the apparent length of the evolution period is limited to $2T$, which limits the final resolution obtainable. However, use of the modern maximum entropy type processing methods may significantly alleviate this problem [9]. As an example, fig.3 shows the ^{1}H-^{31}P correlation spectrum of the synthetic DNA oligomer $d(TA)_{20}$. Although both the ^{1}H and the ^{31}P linewidths are relatively broad for this large fragment, a clear correlation for both ^{31}P resonances is observed. This spectrum confirms the previously made assignment of the ^{31}P resonances, made by comparison with two phosphorothioate analogues [10].

3. DISCUSSION

We have shown that the ^{1}H-^{31}P correlation method can be applied successfully to the study of DNA fragments of a significant size, requiring only moderate NMR sample quantities. Not only does this type of experiment make it possible to obtain an unambiguous assignment of the ^{31}P spectrum, important for structural studies, but it also

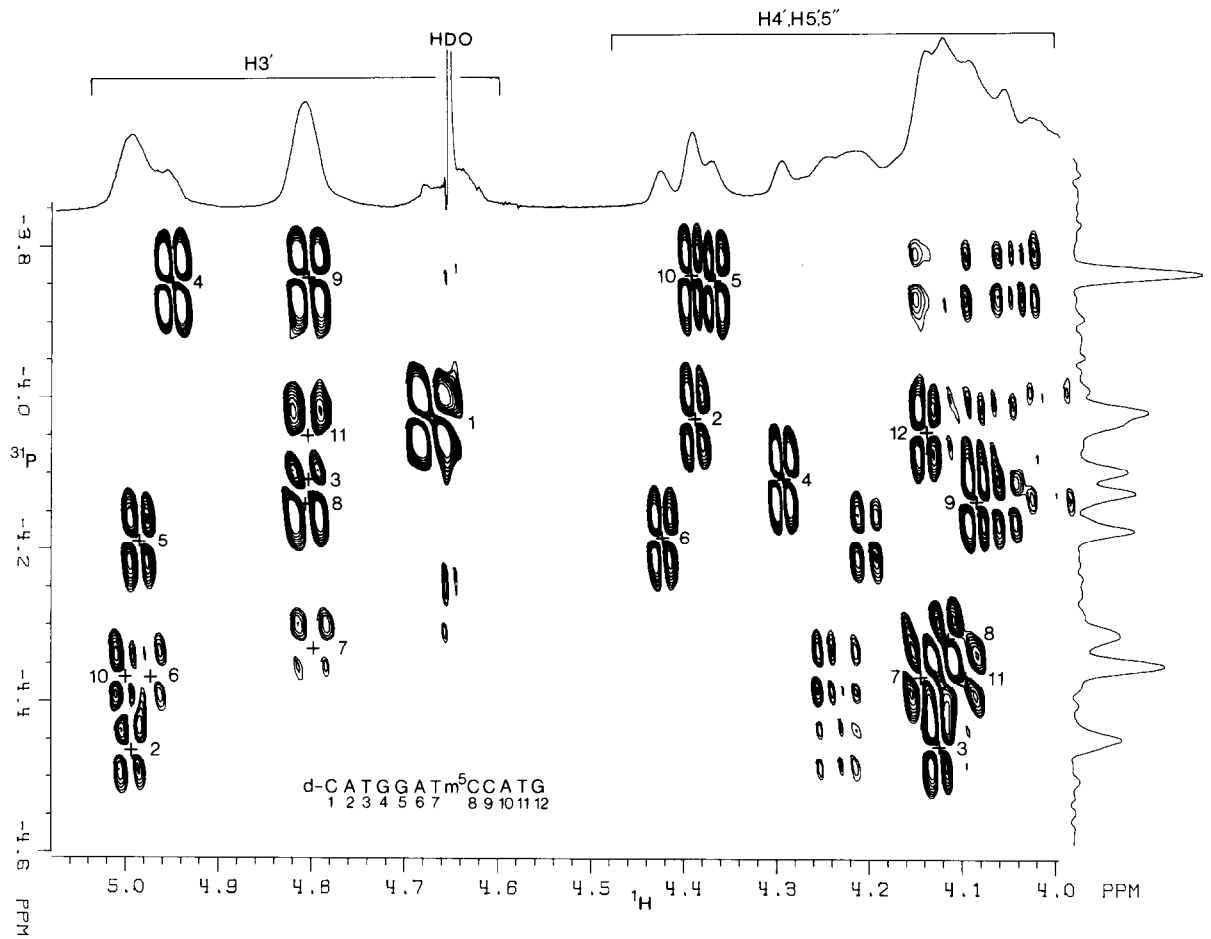

Fig.2. 2D absorption mode ^{1}H-^{31}P correlation spectrum of the dodecamer d(CATGGATm^{5}CCATG), recorded with the scheme of fig.1a. ^{1}H and ^{31}P chemical shifts are relative to TSP and TMP, respectively. The spectrum results from a $2 \times 64 \times 1024$ data matrix with 80 scans per t_1 value. Acquisition times in the t_1 and t_2 dimension are 0.128 and 1.02 s, respectively. The total measuring time is 8 h. Both negative and positive contours are shown and resolution-enhanced 1D ^{1}H and ^{31}P spectra are shown along the two axes of the 2D spectrum. 8 Hz Gaussian broadening is used in the t_2 dimension and 9 Hz exponential narrowing followed by 12 Hz Gaussian broadening is used in the t_1 dimension. The centers of the correlation multiplets are marked + and only correlations with H3′ and H4′ protons are labeled.

provides a means to make sequence-specific resonance assignments independent of an assumed structure of the DNA fragment. The high intensity of the H4′-P correlations suggests a coupling constant larger than the 2 Hz previously estimated [11]. The relatively long transverse relaxation time of the C4′ protons also contributes favorably to the intensity of these cross-peaks.

4. EXPERIMENTAL

The dodecamer was synthesized manually by the solid-phase phosphite triester method using the *O*-*β*-cyanoethylphosphoramidites. 600 A_{260} units were dissolved in 0.4 ml D_2O, containing 0.1 M NaCl, 10 mM sodium phosphate, p^{2}H 7.4. The spectrum of fig.2 was recorded at 35°C.

The 5′-dimethoxytrityl (DMT) derivative of the self-complementary 40-mer, d(TA)$_{20}$, was synthesized on a 2×1-μmol scale using a previously described [12] automated (Applied Biosystems model 380B) version of the phosphoramidite coupling method, with the exceptions that *O*-*β*-cyanoethylamidites were employed, and deprotec-

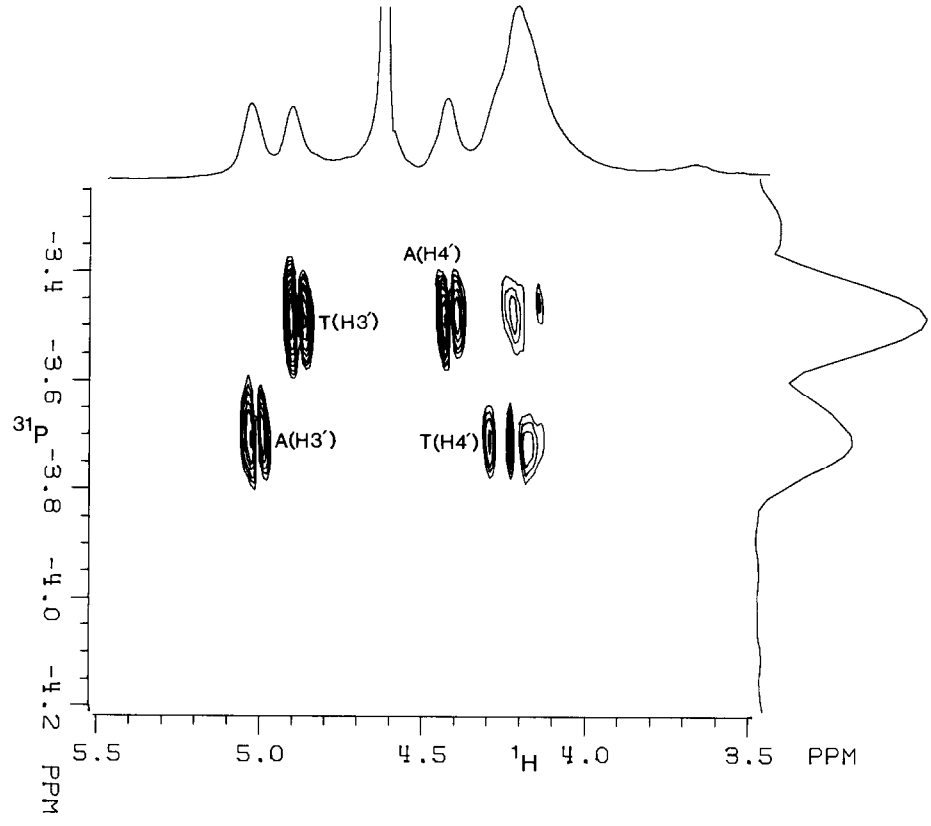

Fig.3. 2D correlation spectrum of the 40-mer d(TA)$_{20}$, recorded with the scheme of fig.1b. The spectrum is absorptive in the ^{1}H dimension but in the absolute value mode in the ^{31}P dimension. ^{1}H and ^{31}P chemical shifts are relative to TSP and sodium phosphate, respectively. The spectrum results from a 2 × 50 × 512 data matrix, with the effective t_1 acquisition period ranging from −25 to +25 ms and the t_2 acquisition period from 0 to 108 ms. The ^{31}P T_1 (1.4 s) was shorter than the ^{1}H T_1 (≈2 s), and a 2-s delay between scans was used. The total measuring time was 12 h. The resolution-enhanced ^{1}H spectrum and the projection of the 2D spectrum on the ^{31}P axis are shown along the sides of the 2D spectrum.

tion with thiophenol-triethylamine was omitted. The crude 5′-DMT material obtained from the parallel syntheses was pooled and dissolved in 12 ml of 0.1 M triethylammonium acetate (pH 7, TEAA). Four equal-volume portions of the resultant solution were each eluted from a reversed-phase HPLC column (Hamilton PRP-1, 7 × 305 mm) with a 1%/min gradient of acetonitrile in 0.1 M TEAA (pH 7) that began and ended at acetonitrile-TEAA ratios of 20:80 and 30:70, respectively [13]. The center-cut fractions which were collected at 10.5–11.5 min were pooled, lyophilized, detritylated, lyophilized, dissolved in 1 ml of 0.2 M NaCl, and then eluted with water from a size-exclusion column (Sephadex G-25M PD-10) to yield the sodium form of d(TA)$_{20}$ (105 A_{260} units, 12.5% yield based on support-bound nucleoside). The sample was dissolved in 0.4 ml D_2O, containing 0.1 M NaCl, 10 mM sodium phosphate, p^{2}H 7.2. The NMR spectrum was recorded at 45°C.

NMR spectra were recorded on a modified Nicolet NT-500 spectrometer equipped with a Cryomagnet Systems 5-mm ^{1}H probe that has a broad-band (^{15}N-^{31}P) decoupling coil for irradiation of low-gamma nuclei.

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